INFECTION AND IMMUNITY, Jan. 2005, p. 277–286 Vol. 73, No. 1
0019-9567/05/$08.00+0 doi:10.1128/IAI.73.1.277–286.2005

Depletion of Complement Has Distinct Effects on the Primary and Secondary Antibody Responses to a Conjugate of Pneumococcal Serotype 14 Capsular Polysaccharide and a T-Cell-Dependent Protein Carrier

Samuel T. Test,* Joyce K. Mitsuyoshi, and Yong Hu

Children's Hospital Oakland Research Institute, Oakland, California

Received 26 July 2004/Returned for modification 2 September 2004/Accepted 24 September 2004

Complement activation plays a critical role in the immune response to T-cell-dependent and T-cell-independent antigens. However, the effect of conjugation of T-cell-dependent protein carriers to T-cell-independent type 2 antigens on the requirement for complement in the humoral immune response to such antigens remains unknown. We studied the role of complement activation on the antibody response of BALB/c mice immunized with the T-cell-independent type 2 antigen serotype 14 pneumococcal capsular polysaccharide (PPS14), either in unmodified form or conjugated to ovalbumin (OVA). In mice immunized with either PPS14 or PPS14-OVA, depletion of endogenous complement at the time of primary immunization by treatment with cobra venom factor (CVF) diminished serum anti-PPS14 concentrations after primary immunization but enhanced antibody responses after secondary immunization. The secondary immunoglobulin G (IgG) anti-PPS14 antibody response after immunization with PPS14-OVA was especially enhanced by complement depletion, was observed at doses as low as 0.2 μg of antigen, and was maximal when CVF was administered within 2 days of immunization. The avidity and opsonophagocytic functions of IgG anti-PPS14 antibodies were comparable in mice immunized with PPS14-OVA with or without complement depletion. Serum anti-PPS14 antibody concentrations were near normal, and the enhancing effects of CVF treatment on the secondary anti-PPS14 antibody response were also apparent in splenectomized mice immunized with PPS14-OVA. These results demonstrate that complement activation can have distinct effects on the primary and secondary antibody responses to a T-cell-independent type 2 antigen, either unmodified or conjugated to a T-cell-dependent protein carrier. These differences should be taken into consideration when using complement to modulate the immune response to vaccines.

Disease caused by *Streptococcus pneumoniae* is a major public health concern throughout the world, with the very young, the elderly, and immunocompromised individuals being particularly susceptible to infection (59). In the United States, *S. pneumoniae* causes more cases of meningitis and pneumonia than any other species of bacteria, resulting in over 7,000 fatalities each year (2). Worldwide, pneumococcal infections result in the death of over one million children annually, primarily in developing countries (25, 48, 59). Local infections in the upper respiratory tract, including sinusitis, bronchitis, and otitis media, are also associated with significant morbidity, and over seven million cases of otitis media alone occur in the United States each year (23). Protective immunity is mediated by antibodies against capsular polysaccharide epitopes and, based on differences in polysaccharide structure, there are over 90 different pneumococcal serotypes. Clinical disease is associated with a variety of serotypes, but the majority of invasive disease worldwide is caused by 11 serotypes (21).

Pneumococcal capsular polysaccharides are classified as T-cell-independent type 2 (TI-2) antigens, which are characterized by high molecular weight, multiple repeat epitopes, persistence in vivo, a failure to stimulate major histocompatibility complex type II-mediated T-cell help, and poor immunogenicity in children under 18 months of age (12, 33). The currently licensed 23-valent pneumococcal capsular polysaccharide vaccine is effective in the majority of adults but is poorly immunogenic in children under 2 years of age and in patients with immunodeficiencies (59). To overcome this lack of efficacy, T-cell-dependent (TD) protein carriers have been coupled to capsular polysaccharides and incorporated into newer pneumococcal vaccines (28). Use of a recently licensed heptavalent pneumococcal conjugate vaccine has resulted in a significant decline in the rate of invasive pneumococcal infections in children in the United States and a significant but more modest decline in disease rates in adults (56).

The capsular polysaccharide of serotype 14 *S. pneumoniae* (PPS14) is included in the currently licensed conjugate vaccine and all others currently under development, as it is one of the three most prevalent serotypes causing invasive pneumococcal disease worldwide (47). PPS14 activates the alternative pathway of complement (19), and its ability to induce a primary antibody response in BALB/c mice is complement dependent (30). However, the role of complement activation in the antibody response to PPS14 conjugated to a TD protein carrier has not been examined. We have used ovalbumin (OVA) as a model TD protein carrier because the murine immune response to OVA has been well characterized and because its low molecular mass (43 kDa) facilitates the preparation of PPS14-OVA conjugates that are free of unconjugated carrier protein. Our previous studies comparing PPS14-OVA and PPS14-C3d con-

* Corresponding author. Mailing address: Children's Hospital Oakland Research Institute, 5700 Martin Luther King, Jr. Way, Oakland, CA 94609-1673. Phone: (510) 450-7630. Fax: (510) 450-7910. E-mail: stest@chori.org.

jugates have shown that PPS14-OVA is a potent immunogen in BALB/c mice (51).

For the studies described here, we wished to inhibit activation of endogenous complement independently at the time of either primary or secondary immunization. Because this is difficult or impossible using C3 or complement receptor knockout mice, we treated mice with cobra venom factor (CVF) to deplete serum complement prior to immunization. CVF is a functional analogue of C3b, but it is not susceptible to degradation and inactivation by factors H and I (55). Hence, injection of mice with CVF results in unregulated complement activation and temporary depletion of serum C3. Our results show that complement depletion at the time of primary immunization of mice with PPS14 or PPS14-OVA results in a suboptimal primary anti-PPS14 antibody response but enhances the secondary (memory) antibody response. When complement is depleted at the time of secondary immunization, less-pronounced effects on the subsequent anti-PPS14 antibody response are observed and consist primarily of decreased anti-PPS14 immunoglobulin G (IgG) concentrations.

MATERIALS AND METHODS

Reagents. OVA (Pierce, Rockford, Ill.) was treated with 10 mM iodoacetamide to block residual thiols, and OVA monomers were isolated by passage over a Bio-Gel P-60 column. Purified PPS14 was purchased from the American Type Culture Collection, Manassas, Va. Purified pneumococcal cell wall polysaccharide (C-PS) was from Statens Serum Institut, Copenhagen, Denmark. C-PS was activated with 1-cyano-4-dimethylaminopyridium tetrafluoroborate (CDAP; Research Organics, Cleveland, Ohio) and biotinylated using biotin-LC-hydrazide (Pierce). Murine anti-PPS14 monoclonal antibodies 44.2 (IgG1, κ) and 9.2 (IgM, κ) were provided by Alexander H. Lucas (Children's Hospital Oakland Research Institute). Baby rabbit serum (as a source of complement) was purchased from CedarLane Laboratories (Hornby, Ontario, Canada).

PPS14 conjugated to OVA was prepared as described previously (51) by using CDAP. Conjugates were isolated by chromatography on a 1- by 50-cm column of Bio-Gel P-300 (Bio-Rad Laboratories). PPS14-OVA preparations were dialyzed into phosphate-buffered saline (PBS) and 0.2-μm filtered before storage at 4°C. The PPS14 concentration of conjugate preparations was determined by a resorcinol sulfuric acid micromethod (35), and the protein concentration was determined with the bicinchoninic acid protein assay (Pierce).

Mice and immunizations. Female BALB/c mice were obtained from Charles River Laboratories (Hollister, Calif.) and were used at 10 to 11 weeks of age. Female BALB/c mice splenectomized or sham-splenectomized at the age of 4 weeks were also purchased from Charles River Laboratories and were rested for 6 weeks prior to immunization. Animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee. Mice were immunized with 1 μg of PPS14 as unmodified PPS14 or PPS14-OVA diluted in 200 μl of sterile, endotoxin-free PBS (Sigma Chemical Co., St. Louis, Mo.). In some experiments, mice were immunized with 0.2 or 0.5 μg of PPS14-OVA. Antigen was administered by subcutaneous injection, with the total dose divided equally between two sites. A second identical injection was given 42 days after the first. Blood samples were obtained 3 days before and at 10, 25, and 39 days after primary immunization and at days 10 and 25 after secondary immunization.

Mice that received CVF were given three intraperitoneal injections of 5 μg of CVF diluted in 200 μl of sterile, endotoxin-free PBS at 28, 24, and 4 h prior to immunization as previously described (30). This treatment regimen completely depleted serum alternative pathway hemolytic activity at the time of immunization as determined using rabbit erythrocytes as alternative pathway activators. Groups of 8 to 10 mice were immunized with or without pretreatment with CVF at the time of primary immunization or at the time of secondary immunization. In experiments to determine the effects of altering the time of CVF treatment relative to the time of immunization with PPS14-OVA, different groups of mice were treated with CVF such that the last dose of CVF was given on the day of immunization, at 2 and 4 days before immunization, and at 2 and 4 days after immunization.

ELISAs for determination of anti-PPS14 antibody concentrations. Serum anti-PPS14 antibodies were measured using enzyme-linked immunosorbent assays (ELISAs) specific for anti-PPS14 IgM and anti-PPS14 IgG. Purified 9.2 IgM and 44.2 IgG anti-PPS14 monoclonal antibodies were used as standards in the assays. Wells of microtiter plates (Nunc MaxiSorp ImmunoPlate; Nalge Nunc International Corp., Rochester, N.Y.) were coated overnight with 100 μl of rabbit antiserum against PPS14 (Statens Serum Institut) diluted 1/1,000 in PBS containing 0.02% sodium azide. The coating antibody was aspirated, and wells were incubated with 200 μl of PBS containing 1% bovine serum albumin (BSA) and 0.1% sodium azide (PBS-BSA-Az) for 1 h at room temperature. Plates were washed with PBS containing 0.1% Tween 20 (Bio-Rad), and 100 μl of a saturating concentration (10 μg/ml) of purified PPS14 in PBS containing 0.05% Tween 20 was added to each well. Plates were incubated overnight at 4°C and then washed. Fifty microliters of PBS-BSA-Az containing 20 μg of C-PS (PBS-BSA-Az plus C-PS)/ml was added to wells, and 50 μl of PBS-BSA-Az plus C-PS containing 20 μg of PPS14/ml was added to a duplicate set of wells. Fifty microliters of serum sample diluted in PBS-BSA-Az plus C-PS (three or four dilutions of each sample) or standard was added to each set of wells, and plates were incubated for 2 h at 37°C. The plates were washed and incubated for 90 min at 37°C with biotinylated rabbit anti-mouse IgM or IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.) followed by washing and incubation for 60 min at 37°C with streptavidin-alkaline phosphatase conjugate (Caltag Laboratories, Burlingame, Calif.). After a final wash, plates were developed with Sigma 104 phosphatase substrate, and the absorbance at 405 nm was determined on a SpectraMax 340 microplate reader (Molecular Devices Corp., Sunnyvale, Calif.). Antibody concentrations were calculated after subtracting the absorbance reading for wells containing 20 μg of PPS14/ml from those containing diluted sample alone. The minimal detectable concentration was 0.19 ng/ml for the IgM anti-PPS14 ELISA and 0.25 ng/ml for the IgG anti-PPS14 ELISA.

ELISA for determination of anti-OVA IgG antibody concentrations. Serum anti-OVA IgG concentrations were determined by ELISA using methods identical to those used to measure IgG antibodies against PPS14, except that plates were coated with 100 μl of OVA (Pierce)/well at 2 μg/ml and purified monoclonal anti-OVA IgG (Sigma) was used as a standard.

Isotype analysis. The IgG and IgA subclass compositions of serum antibodies to PPS14 were determined by ELISA with the SBA clonotyping system/AP and murine IgG subclass standards (Southern Biotechnology Associates, Birmingham, Ala.). Serum samples were diluted in PBS containing 1% BSA, 0.1% sodium azide, and 20 μg of pneumococcal cell wall polysaccharide/ml (dilution buffer) such that the anti-PPS14 IgG concentration was identical in each sample. A control for each serum sample consisted of diluted serum (or standard) plus an equal volume of PPS14 (20 μg/ml) in dilution buffer. The value for this control was subtracted from the corresponding value for diluted serum incubated with an equal volume of dilution buffer. PPS14-specific IgA, IgG1, IgG2a, IgG2b, and IgG3 were determined using alkaline phosphatase goat anti-mouse conjugates specific for each subclass. Results are expressed as the absorbance at 405 nm at 2 h for each serum sample.

IgG purification. IgG was purified from five 25-day post-secondary immunization serum samples each from untreated and CVF-treated mice immunized with PPS14-OVA. Serum IgG was purified by affinity chromatography on 1-ml HiTrap protein G columns (Amersham Biosciences, Uppsala, Sweden). Following elution from the protein G column, fractions containing IgG were pooled and concentrated on a Centricon filtration device with a YM10 membrane (Millipore, Bedford, Mass.). The concentrated IgG was dialyzed into PBS, and sufficient 5% BSA was added to give a final concentration of 0.5% BSA.

To remove serum anti-C-PS antibody, Dynabeads M-280 streptavidin (Dynal Biotech, Oslo, Norway) were incubated with biotinylated C-PS to generate C-PS-coated magnetic beads. Fifty microliters of Dynabeads M-280 streptavidin was coated with biotinylated C-PS and washed with PBS plus 0.5% BSA. The IgG preparation was immediately added to the C-PS beads and incubated with gentle mixing for 2 h at 4°C, and the supernatant was collected. To eliminate any residual IgM, the resulting IgG preparation was incubated as above with Dynabeads M-450 coated with rat anti-mouse IgM (Dynal). After this purification procedure, anti-PPS14 IgG recovery averaged 90.1% and anti-PPS14 IgM averaged 0.2% of total Ig. IgG3 anti-PPS14 was lost during the purification procedure, so the final IgG preparation consisted almost solely of IgG1 anti-PPS14.

Anti-PPS14 IgG avidity assays. Avidity was measured according to previously published methods (37) which have been used to measure avidity of antibodies to both pneumococcal (8) and nonpneumococcal (36) polysaccharides. Polystyrene 96-well plates (Nunc) were coated overnight at 4°C with 1 μg of PPS14/ml. The plates were blocked with PBS-BSA-Az, followed by washing with 0.1% Tween 20 in PBS. Purified IgG samples were diluted in PBS-BSA-Az plus C-PS to a final anti-PPS14 IgG concentration of 50 ng/ml. Equal volumes of purified IgG and buffer or PPS14 at 10 different concentrations, ranging from 0.2 to 20,000 ng/ml in PBS plus 0.02% azide, were mixed in 12- by 75-mm tubes, and then 100 μl of the mixture was added to wells, incubated at 37°C for 2 h, and

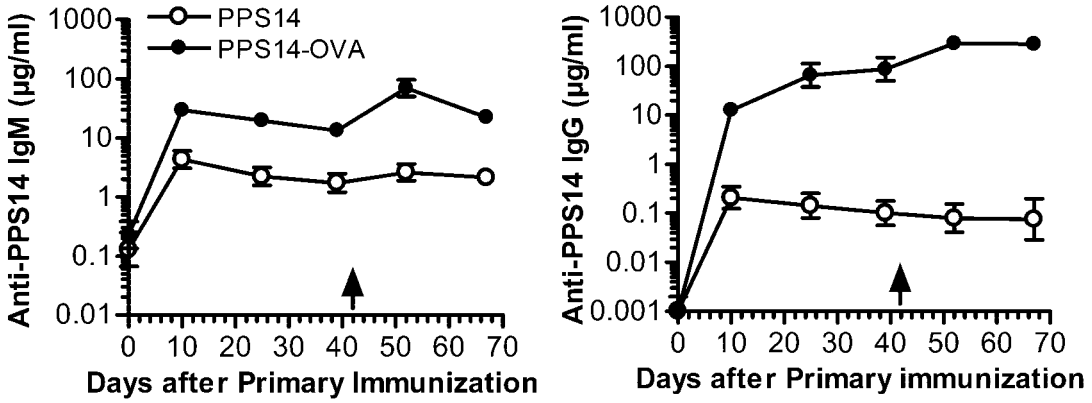

FIG. 1. Enhancement of the anti-PPS14 antibody response by conjugation of OVA to PPS14. BALB/c mice (10 mice per group) were immunized subcutaneously with 1 μg of PPS14 as either unmodified PPS14 or PPS14-OVA. A second injection identical to the first was given 42 days later. Serum anti-PPS14 IgM (left) and IgG (right) GMC are shown for serum samples obtained 3 days before and 10 and 25 days after each injection. Error bars represent the 95% confidence interval. Arrows indicate the day of secondary immunization. *P* values for mice immunized with PPS14-OVA versus mice immunized with PPS14 were highly significant ($P \leq 10^{-8}$) for all postimmunization bleeds for both IgM and IgG anti-PPS14.

washed with PBS plus 0.1% Tween 20. The remainder of the assay was performed as described for the anti-PPS14 IgG ELISA. An avidity constant (αK) was calculated as the inverse molar concentration of soluble PPS14 required to inhibit antibody binding to solid-phase PPS14 by 50%, assuming a molecular mass of 1,000,000 Da for PPS14.

In addition, we measured direct binding of anti-PPS14 IgG to wells coated with seven different concentrations of PPS14 ranging from 1 to 1,000 ng/ml. The PPS14 coating concentration at which anti-PPS14 IgG binding was 50% of maximum was calculated for each IgG preparation.

Opsonophagocytic killing assay. Killing of opsonized serotype 14 pneumococci by RAW 264.7 murine macrophages was assayed according to previously published methods (46) modified as needed for analysis of mouse anti-PPS14 IgG. In the wells of 96-well microplates, 10 μl (1,000 CFU) of serotype 14 pneumococci in the third log phase was mixed with 20 μl of purified IgG from mouse serum and sufficient Hanks balanced salt solution plus calcium and magnesium ($HBSS^{2+}$) plus 0.1% gelatin to achieve a total volume of 50 μl. Final IgG concentrations (after addition of phagocytes) ranged from 0.2 to 2,000 ng/ml. The microplate was placed in 5% CO_2 at 37°C for 15 min. Five microliters of baby rabbit serum as a source of complement was added, followed immediately by 50 μl of RAW 264.7 cells (45) at 8×10^6/ml. Control wells contained bacteria, complement, and RAW 264.7 cells but no IgG. The microplate was placed on an orbital rotator at 200 rpm and incubated at 37°C for 45 min. Twenty-five microliters was removed from each well and added to 300 μl of $HBSS^{2+}$, and 162.5 μl was plated on blood agar plates (trypticase soy agar with 5% sheep blood; Becton Dickinson and Co., Sparks, Md.) in duplicate. The plates were cultured overnight at 37°C in 5% CO_2, and bacterial colonies were counted the following morning. Based on the average number of CFU, killing was calculated according to the following formula: percent killing = 100 × (1 − [CFU of test IgG]/[CFU of the complement + RAW 264.7 control]).

Statistical analysis. Serum anti-PPS14 IgM and IgG concentrations were determined for individual mice within each immunization group, and the geometric mean and 95% confidence intervals of the geometric mean were calculated. To eliminate the effects of mouse-to-mouse variability, statistical comparisons were made on log-transformed data. Comparisons between mice vaccinated without pretreatment and those receiving CVF were done using Student's *t* test for unpaired samples. Statistical significance was set at a *P* level of ≤0.05.

RESULTS

Comparison of the anti-PPS14 antibody response after immunization with unmodified PPS14 and PPS14-OVA. In our original experiments comparing the anti-PPS14 antibody response to PPS14 and PPS14 conjugates, serum anti-PPS14 concentrations were measured by radioantigen binding assay (RABA) (51). Subsequent to that publication, murine IgM and IgG monoclonal antibodies against PPS14 became available to us. These were used as standards in ELISAs we established to measure both IgM and IgG anti-PPS14 concentrations. Figure 1 and Table 1 summarize data comparing serum anti-PPS14 antibody concentrations for mice immunized with unmodified PPS14 or PPS14-OVA. Conjugation of OVA to PPS14 markedly enhanced the immunogenicity of the polysaccharide after both primary and secondary immunization. Maximum anti-PPS14 IgM concentrations were achieved 10 days after secondary immunization and were increased 25-fold for mice immunized with PPS14-OVA compared with mice immunized with PPS14. The magnitude of the IgG anti-PPS14 antibody response was enhanced even more by conjugation of OVA. Mice immunized with PPS14 showed no boost in anti-PPS14 IgG after secondary immunization, with serum concentrations reaching 0.08 μg/ml at 10 days after secondary immunization. By contrast, mice immunized with PPS14-OVA had a threefold boost in anti-PPS14 IgG after secondary immunization and achieved maximal anti-PPS14 IgG concentrations of 292.97 μg/ml, over 3,600 times the concentrations achieved after immunization with PPS14.

Effect of complement depletion on the anti-PPS14 antibody response to PPS14 and PPS14-OVA. To determine the role of endogenous complement in the humoral immune response to PPS14 and PPS14-OVA, mice were treated with three intraperitoneal injections of 5 μg of cobra venom factor (CVF) at 28, 24, and 4 h prior to either primary or secondary immunization. The results of this experiment are summarized in Table 1. Anti-PPS14 IgM and IgG concentrations at 10 days after primary immunization were decreased by treatment with CVF, and the decreases were highly significant, except for anti-PPS14 IgM concentrations in mice immunized with PPS14. Anti-PPS14 IgM concentrations remained low at 25 and 39 days after primary immunization, never exceeding 28% of levels seen in control mice (data not shown). Although anti-PPS14 IgG concentrations increased slightly from day 10 to days 25 and 39, they too remained low and did not exceed 4% of concentrations observed in control mice (data not shown). Treatment with CVF at the time of secondary immunization

TABLE 1. Effect of CVF on the anti-PPS14 antibody response to PPS14 and PPS14-OVA[a]

Immunization	Anti-PPS14 response (μg/ml)			
	Postprimary		Postsecondary	
	IgM	IgG	IgM	IgG
PPS14, no CVF	4.33 (3.09–6.06)	0.21 (0.12–0.35)	2.61 (1.88–3.61)	0.08 (0.04–0.15)
PPS14, CVF pre-1°	3.59 (2.51–5.13); $P = 0.47$	0.01 (0.00–0.02); **$P = 0.00004$**	21.78 (14.89–31.84); **$P = 10^{-7}$**	0.96 (0.56–1.64); **$P = 0.00002$**
PPS14, CVF pre-2°			4.15 (2.50–6.90); $P = 0.15$	0.05 (0.02–0.12); $P = 0.39$
PPS14-OVA, no CVF	29.63 (23.88–36.76)	12.67 (8.88–18.08)	69.80 (50.39–96.69)	292.97 (221.76–387.03)
PPS14-OVA, CVF pre-1°	5.02 (3.35–7.53); **$P = 3 \times 10^{-7}$**	0.31 (0.18–0.52); **$P = 7 \times 10^{-10}$**	154.16 (108.06–219.93); **$P = 0.004$**	782.38 (362.00–1,690.95); **$P = 0.02$**
PPS14-OVA, CVF pre-2°			66.49 (48.45–91.26); $P = 0.84$	167.76 (93.76–300.16); $P = 0.11$

[a] Groups of 10 BALB/c mice were immunized subcutaneously with 1 μg of PPS14 as PPS14 or PPS14-OVA. A second 1-μg immunization was given 42 days after the first. Serum anti-PPS14 IgM and IgG GMC values are shown for 10 days post-primary and 10 days post-secondary immunization for mice receiving no CVF, CVF at the time of primary immunization (pre-1°), or CVF at the time of secondary immunization (pre-2°). The 95% confidence interval is shown in parentheses; for mice receiving CVF, the *P* value (versus mice not receiving CVF) is shown. Statistically significant *P* values are highlighted in boldface type.

also resulted in decreases in anti-PPS14 antibody concentrations at 10 days after immunization, although the effect was more modest and did not reach statistical significance. Surprisingly, treatment with CVF at the time of primary immunization with PPS14 resulted in a significant increase in anti-PPS14 antibody concentrations after secondary immunization. At 10 days after secondary immunization, anti-PPS14 IgM was 8 times greater in mice receiving CVF compared with untreated mice and anti-PPS14 IgG was 12 times greater, although the latter constituted a small proportion of the total antibody response. In mice immunized with PPS14-OVA, anti-PPS14 antibody concentrations after secondary immunization were also significantly increased in CVF-treated mice to levels 2.7 times those seen in control mice. Whereas the maximum anti-PPS14 IgG concentration in mice immunized without CVF treatment was 685 μg/ml, 6 of 10 mice treated with CVF before immunization with PPS14-OVA had anti-PPS14 IgG concentrations of >750 μg/ml, and 3 of the mice had concentrations of >2,650 μg/ml. Of note, the 489-μg/ml absolute increase in geometric mean concentration (GMC) of serum anti-PPS14 IgG after immunization with PPS14-OVA plus CVF versus that following PPS14-OVA alone exceeded the absolute increase of 293 μg/ml that resulted from conjugation of OVA to PPS14. We also examined sera for the presence of OVA-specific IgG antibodies. In contrast to the anti-PPS14 response, the anti-OVA response was extremely weak and variable and, therefore, we could see no clear effect of complement depletion. For example, at 10 days after secondary immunization, GMC anti-PPS14 IgG was ~0.8 μg/ml in both control and CVF-treated mice. The low anti-OVA response probably was a consequence of the low dose of OVA (~1.5 μg) in each injection of PPS14-OVA conjugate and the absence of adjuvant in our vaccine preparations.

Effect of complement depletion on the anti-PPS14 antibody response after immunization with lower doses of PPS14-OVA. We performed a number of additional experiments to further characterize the effect of CVF treatment at the time of primary immunization on the anti-PPS14 antibody response to PPS14-OVA. To determine if the effects of CVF treatment on the secondary anti-PPS14 antibody response would be maintained at lower doses of conjugate, we examined the effects of CVF administered at the time of primary immunization in mice immunized with two injections of 0.2, 0.5, or 1.0 μg of PPS14-OVA (Fig. 2). Similar to its effects after immunization with 1.0 μg of conjugate, CVF caused decreases in anti-PPS14 IgM and IgG 10 days after primary immunization with either 0.2 or 0.5 μg of PPS14-OVA and marked increases in anti-PPS14 Ig at 10 days after secondary immunization, which were highly significant for anti-PPS14 IgG (Fig. 2). The effect of CVF was especially pronounced after immunization with 0.2 μg of PPS14-OVA, where the serum anti-PPS14 IgG GMC for mice treated with CVF (1,042.59 μg/ml) was over 10 times the value for mice immunized with conjugate alone (92.77 μg/ml).

Timing of treatment and the effect of CVF on the anti-PPS14 antibody response after immunization with PPS14-OVA. When CVF is given to BALB/c mice in multiple intraperitoneal injections over a 24-h period, serum C3 levels reach a nadir of ≤3% of pretreatment C3 levels within 6 to 8 h after the last injection (31), remain low until about day 4 (31, 40), and then return to pretreatment levels over another 2 to 4 days (31, 40). To determine the effects of altering the time of CVF treatment relative to the time of immunization, mice were treated with CVF at the time of immunization, at 2 and 4 days before immunization, and at 2 and 4 days after immunization (Fig. 3). At 10 days after primary immunization, GMC anti-PPS14 IgM and IgG values were significantly decreased compared to untreated controls for mice treated with CVF 2 days before ($P = 0.003$ for IgM anti-PPS14; $P = 0.0003$ for IgG anti-PPS14) and at the time of immunization ($P = 0.002$ for IgM anti-PPS14; $P = 0.00004$ for IgG anti-PPS14), but not for mice treated with CVF 4 days before or 2 or 4 days after immunization. At 10 days after secondary immunization, anti-PPS14 IgM concentrations were significantly increased for mice treated with CVF 2 days before ($P = 0.02$) or at the time of primary immunization ($P = 0.03$), but not at the other times. Anti-PPS14 IgG concentrations were significantly increased only for mice receiving CVF at the time of primary immunization ($P = 0.04$), although modest increases in anti-PPS14 IgG were observed in mice receiving CVF 2 days before or after immunization. Thus, it appears that complement must be

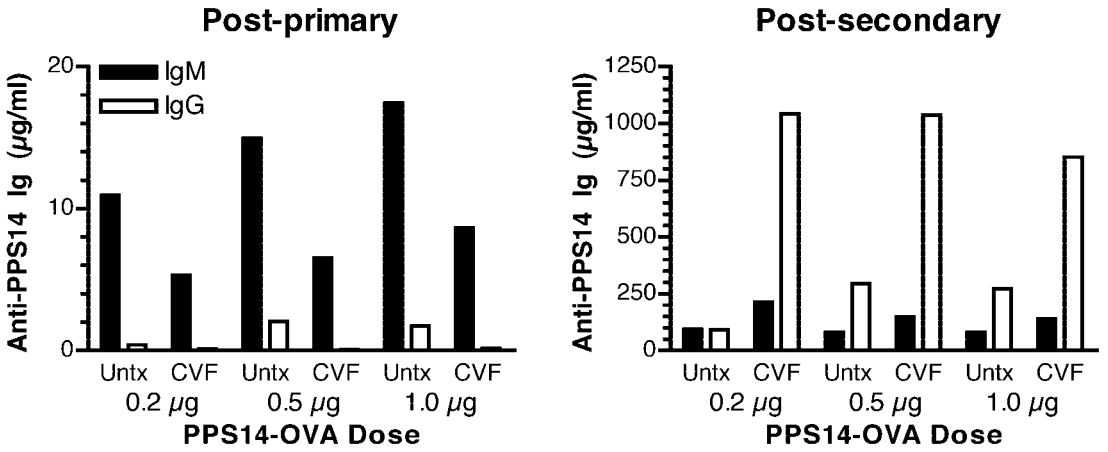

FIG. 2. Effect of CVF treatment on the anti-PPS14 antibody response in mice immunized with different doses of PPS14-OVA. Groups of eight BALB/c mice were immunized subcutaneously with 0.2, 0.5, or 1.0 μg of PPS14 as PPS14-OVA. A second immunization at the same dose was given 42 days after the first. Bars represent the serum anti-PPS14 IgM and IgG GMC values at 10 days post-primary (left) and 10 days post-secondary (right) immunization for untreated mice (Untx) or mice receiving CVF at the time of primary immunization. At 10 days after secondary immunization, anti-PPS14 IgM was significantly increased in CVF-treated mice compared with untreated mice for animals immunized with 0.2 μg of PPS14-OVA ($P = 0.01$), and anti-PPS14 IgG was significantly increased for mice immunized with 0.2 μg ($P = 0.0007$), 0.5 μg ($P = 0.006$), or 1.0 μg ($P = 0.02$) of PPS14-OVA.

depleted during the first 2 to 4 days after primary immunization with PPS14-OVA to see an effect on the anti-PPS14 memory response.

Effect of complement depletion on the isotype profile of mice immunized with PPS14-OVA. To determine whether the increase in anti-PPS14 Ig concentration resulting from treatment with CVF at the time of primary immunization was accompanied by changes in the isotype distribution of IgG and IgA anti-PPS14 antibodies, we used an isotyping kit from Southern Biotechnology Associates to analyze serum samples collected at 25 days after secondary immunization. Figure 4 shows that as previously reported (51), anti-PPS14 IgG was predominantly of the IgG1 subclass with small amounts of IgG2a, IgG2b, and IgG3. IgA anti-PPS14 was negligible. Treatment with CVF did not alter the amount of IgG1 anti-PPS14 but significantly increased IgG3 anti-PPS14 to 3.6 times the value seen in mice immunized with PPS14-OVA alone. There were modest but insignificant decreases in IgG2a and IgG2b anti-PPS14. IgA anti-PPS14 doubled after CVF treatment but still constituted a negligible proportion of the total anti-PPS14 antibody response. Because of differences in the subclass-specific detecting antibodies used for the isotype analysis, it was difficult to make quantitative comparisons between different IgG subclasses within immunization groups, even though IgG1 clearly was the predominant subclass. To help us make more quantitative comparisons, we determined the concentrations of murine IgG subclass standards that gave equivalent optical density (OD) readings in the isotype assay. We found that a murine

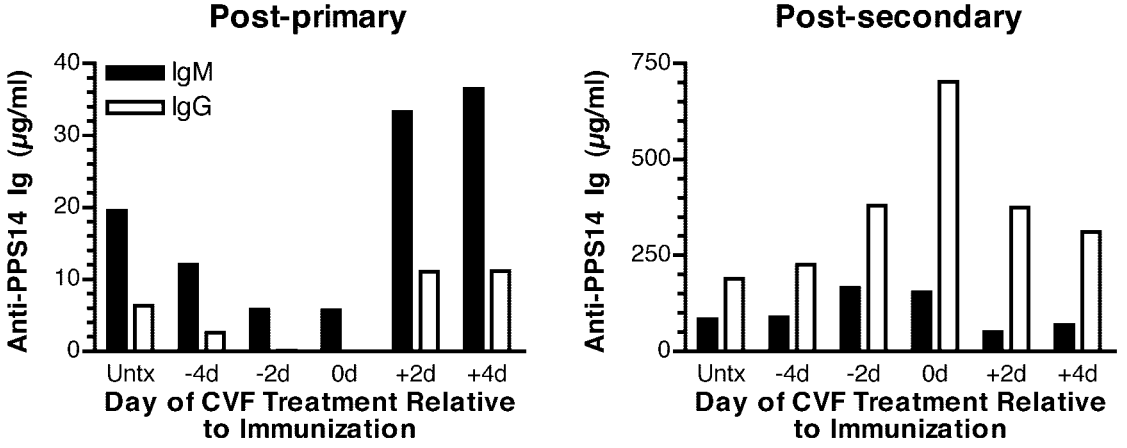

FIG. 3. Effect of treatment with CVF at different times relative to immunization on the anti-PPS14 antibody response in mice immunized with PPS14-OVA. Groups of eight BALB/c mice were immunized subcutaneously with 1 μg of PPS14 as PPS14-OVA. A second immunization at the same dose was given 42 days after the first. CVF (5 μg) was administered by intraperitoneal injection 28, 24, and 4 h prior to primary immunization. The values shown below the *x* axis indicate the day of the last CVF injection relative to the day of immunization. Bars represent serum anti-PPS14 IgM and IgG GMC values at 10 days post-primary (left) and 10 days post-secondary (right) immunization for untreated mice (Untx) or mice receiving CVF at the time of primary immunization.

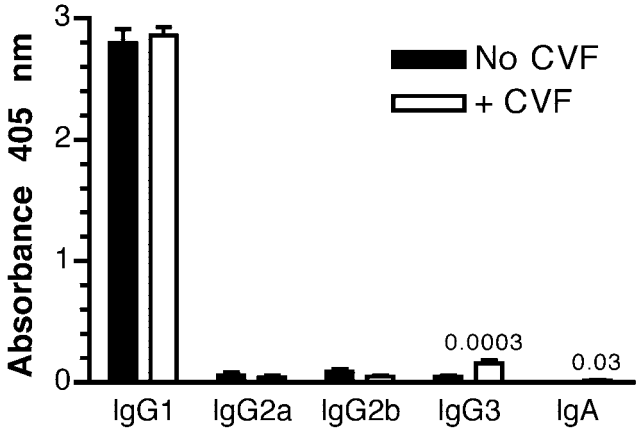

FIG. 4. Effect of CVF treatment on the isotype profile of anti-PPS14 Ig from mice immunized with PPS14-OVA. Serum samples collected at 25 days after secondary immunization from the mice described in the legend to Table 1 were analyzed by ELISA for anti-PPS14 IgG subclass and IgA distribution. Results are expressed as the mean (± standard error) A_{405} at 2 h for 10 serum samples in each immunization group. Where the A_{405} for a given isotype was significantly altered in mice receiving CVF, the *P* value is shown above the relevant bar.

IgG3 concentration 2.9 times that of IgG1 was required to give the same OD reading in the assay. Thus, we estimate that IgG3 anti-PPS14 increased from ~4.6% of the IgG1 anti-PPS14 concentration in serum samples from untreated mice to 16.3% of anti-PPS14 IgG1 in mice treated with CVF.

Effect of complement depletion on functional attributes of IgG anti-PPS14 antibodies from mice immunized with PPS14-OVA. To determine whether the functional attributes of IgG anti-PPS14 antibodies were altered by treatment with CVF, we purified IgG from the same serum samples used for the isotype analysis. IgG was purified from five representative serum samples in each treatment group by passage of serum over Hi-Trap protein G columns (Amersham), followed by incubation with Dynal magnetic beads coated with C-PS to remove antibodies to C-PS and Dynal beads coated with rat anti-mouse IgM to remove any residual IgM. Anti-PPS14 IgG in the final preparation consisted almost solely of the IgG1 subclass. Avidity was measured by two methods. The first was an ELISA-based assay (37) in which different concentrations of soluble PPS14 inhibited binding of anti-PPS14 IgG to solid-phase PPS14. This assay has previously been used to measure avidity of antibodies to pneumococcal polysaccharides (8). An avidity constant (αK) was calculated as the inverse molar concentration of soluble PPS14 required to inhibit IgG binding by 50%. Wells were coated with 1 μg of PPS14/ml. The results of this analysis are shown in Fig. 5A. The mean αK value for untreated mice immunized with PPS14-OVA was 1.53×10^{11} (standard error [SE], 8.38×10^{10}), which was six times the value ($2.54 \times 10^{10} \pm 1.08 \times 10^{10}$) for mice that received CVF at the time of primary immunization. Because of overlap in values for individual mice, the differences were not statistically significant. In the second avidity assay (Fig. 5B), we measured direct binding of anti-PPS14 IgG to wells coated with seven different concentrations of PPS14 ranging from 1 to 1,000 ng/ml. The PPS14 coating concentration at which anti-PPS14 IgG binding was 50% of maximal values was 3.75 ± 0.48 ng/ml for untreated mice and 6.32 ng/ml for mice treated with CVF ($P = 0.002$). However, the magnitude of the difference was very small and may not be biologically important. Nonetheless, these results suggest that complement depletion induces subtle differences in avidity of IgG anti-PPS14 antibodies, with CVF treatment causing a decrease in avidity to soluble PPS14 (Fig. 5A) and only a small decrease in avidity to solid-phase PPS14 (Fig. 5B).

PPS14 in the capsule of viable *S. pneumoniae* probably exists in a state intermediate between that of soluble PPS14 and PPS14

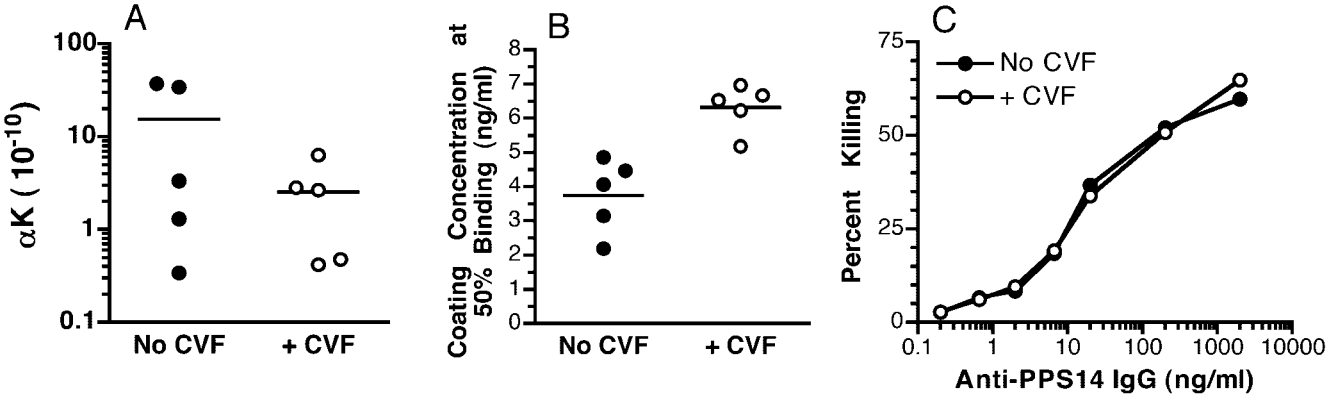

FIG. 5. Functional attributes of anti-PPS14 IgG purified from serum samples collected at 25 days after secondary immunization. For each immunization group, IgG was purified from five of the 25-day postsecondary serum samples used for the isotype analysis described in the legend for Fig. 3. Avidity was determined in a competition binding ELISA (A) and by direct binding to PPS14-coated wells of 96-well plates (B). In panel A, avidity is expressed as an avidity constant (αK), the inverse molar concentration of soluble PPS14 which inhibited binding of anti-PPS14 IgG to solid-phase PPS14 by 50%. In panel B, avidity is expressed as the coating concentration of PPS14 at which anti-PPS14 IgG binding was 50% of maximal values. In panels A and B, symbols represent values for individual IgG preparations and horizontal bars indicate the means. (C) Opsonophagocytic killing of serotype 14 *S. pneumoniae* by mouse RAW 264.7 macrophages was determined by counting surviving colonies grown on blood agar plates after incubation of bacteria, different concentrations of anti-PPS14 IgG, baby rabbit serum as a complement source, and RAW 264.7 cells. Recovery of bacteria in control wells was 135 ± 4 CFU (expected = 125 CFU). Results shown are the mean percentage of serotype 14 *S. pneumoniae* bacteria killed at each concentration of anti-PPS14 IgG. Killing in the absence of complement was assessed at an anti-PPS14 IgG concentration of 20 ng/ml and was 3.5% ± 1.6% ($n = 10$).

TABLE 2. Effect of splenectomy without or with CVF treatment on the anti-PPS14 antibody response to PPS14 and PPS14-OVA

Immunization	Anti-PPS14 response (μg/ml)[a]			
	Postprimary		Postsecondary	
	IgM	IgG	IgM	IgG
Expt 1				
PPS14, sham splenectomized	5.18 (3.12–8.45)	0.28 (0.12–0.66)	3.91 (2.54–6.02)	0.10 (0.03–0.31)
PPS14, splenectomized	0.22 (0.13–0.36); **$P = 0.0000004$**	0.01 (0.01–0.02); **$P = 0.00002$**	1.04 (0.67–1.59); **$P = 0.0008$**	0.05 (0.04–0.06); $P = 0.27$
PPS14-OVA, sham splenectomized	32.43 (21.53–48.84)	10.47 (5.67–19.34)	48.36 (27.13–86.21)	224.21 (181.50–276.97)
PPS14-OVA, splenectomized	3.44 (1.84–6.43); **$P = 0.00004$**	0.74 (0.27–2.06); **$P = 0.001$**	32.55 (14.36–73.77); $P = 0.45$	181.39 (92.31–356.45); $P = 0.57$
Expt 2				
PPS14, no CVF	0.48 (0.23–1.01)	0.01 (0.01–0.02)	1.07 (0.44–2.58)	0.03 (0.02–0.07)
PPS14, CVF pre-1°	1.86 (0.85–4.07); **$P = 0.03$**	0.02 (0.01–0.04); $P = 0.31$	3.09 (1.82–5.27); $P = 0.06$	0.07 (0.02–0.21); $P = 0.31$
PPS14-OVA, no CVF	4.93 (3.55–6.85)	0.77 (0.58–1.01)	17.35 (12.19–24.71)	83.11 (58.66–117.76)
PPS14-OVA, CVF pre-1°	7.53 (5.11–11.08); $P = 0.12$	0.24 (0.10–0.60); **$P = 0.03$**	147.78 (87.76–248.85); **$P = 0.00001$**	394.75 (206.40–755.37); **$P = 0.001$**

[a] In experiment 1, BALB/c mice were splenectomized or sham splenectomized at 4 weeks of age, rested for 6 weeks, and then immunized with 1 μg of PPS14 as PPS14 or PPS14-OVA (eight mice per group). In experiment 2, BALB/c mice were splenectomized at 4 weeks of age, rested for 6 weeks, and then immunized with 1 μg of PPS14 as PPS14 or PPS14-OVA, with or without pretreatment (pre-1°) with CVF (eight mice per group). In each experiment, a second 1-μg immunization was given 42 days after the first. GMC serum anti-PPS14 IgM and IgG values are shown for 10 days post-primary immunization and 10 days post-secondary immunization. The 95% confidence interval is shown within parentheses, and the *P* value is shown for splenectomized mice versus the corresponding group of sham-splenectomized mice in experiment 1 and for CVF-treated splenectomized mice versus splenectomized mice not receiving CVF in experiment 2. Statistically significant *P* values are highlighted in boldface type.

bound to a plastic surface. Thus, we measured the opsonophagocytic activity of the purified IgG preparations by incubating viable bacteria with different concentrations of anti-PPS14 IgG and measuring opsonophagocytic killing by RAW 264.7 murine macrophages using previously published methods (46). As can be seen in Fig. 5C, IgG anti-PPS14 antibodies from mice immunized with PPS14-OVA with or without CVF treatment were equivalent in their ability to support opsonophagocytic killing of bacteria.

In summary, depletion of serum complement at the time of primary immunization with PPS14-OVA resulted in increased concentrations of anti-PPS14 antibodies after secondary immunization and increased IgG3 anti-PPS14, but the IgG1 anti-PPS14 antibodies still constituted the majority of anti-PPS14 IgG and were equally capable of supporting opsonophagocytic killing of serotype 14 *S. pneumoniae* as were their counterparts in mice not depleted of complement. These results suggest that somatic hypermutation and affinity maturation occurring in germinal centers after immunization with PPS14-OVA were similar in complement-depleted and untreated mice.

Effect of splenectomy on the anti-PPS14 antibody response to PPS14 and PPS14-OVA. To determine whether conjugation of OVA affected the ability of splenectomized mice to respond to PPS14, we performed immunizations in mice that had been splenectomized 6 weeks prior to immunization (Table 2, experiment 1). Sham-splenectomized mice underwent an identical surgical procedure, except the spleen was not removed. At 10 days post-primary immunization, there were significant decreases in both IgM and IgG anti-PPS14 in splenectomized mice immunized with either PPS14 or PPS14-OVA. At 10 days after secondary immunization, the magnitude of the differences in the anti-PPS14 response between splenectomized and control mice had narrowed for both vaccine groups and was no longer significantly different for anti-PPS14 IgM in mice immunized with PPS14-OVA and for anti-PPS14 IgG in mice immunized with either PPS14 or PPS14-OVA. Thus, the primary anti-PPS14 antibody response is critically dependent on the presence of a spleen, but the anti-PPS14 memory response after immunization with PPS14-OVA is nearly normal even in the absence of a spleen.

Effect of complement depletion on anti-PPS14 antibody response to PPS14 and PPS14-OVA in splenectomized mice. Since the anti-PPS14 antibody response is essentially normal in splenectomized mice, we were curious whether CVF would have the same effect on the secondary response to PPS14-OVA as observed in normal mice. CVF treatment of splenectomized mice resulted in small changes in already low anti-PPS14 antibody responses after primary immunization (Table 2, experiment 2). After secondary immunization with PPS14-OVA but not PPS14, mice pretreated with CVF at the time of primary immunization had significant increases in anti-PPS14 IgM and IgG compared with untreated mice. The magnitude of the effect was similar or greater than that seen in normal mice (Table 1). Although postsecondary anti-PPS14 IgM and IgG concentrations in splenectomized mice immunized with PPS14-OVA in this experiment were about half those observed in the first experiment shown in Table 2, anti-PPS14 Ig concentrations observed in CVF-treated mice in this experiment were at least twice the values seen for untreated splenectomized mice in the first experiment summarized in Table 2.

Together with the results shown in Tables 1 and 2 (experiment 1), these data suggest the possibility that the memory response to PPS14-OVA develops in a lymphoid compartment distinct from the spleen even when the spleen is present. Thus, complement depletion has a similar effect on the memory response in the presence or absence of the spleen. Still, it remains possible that the memory response normally develops in the spleen, but that responsibility for PPS14-specific memory cell development can be assumed by other secondary lymphoid tissues in the absence of a spleen.

DISCUSSION

Following immunization with a polysaccharide or other TI-2 antigen, complement activation could occur as a consequence of direct activation of the alternative pathway of complement by the antigen or by activation of the classical pathway after binding of preexisting natural IgM antibody or newly generated antigen-specific IgM. Both mechanisms probably play a role in complement activation by PPS14, which previously has been shown to be a weak activator of the alternative pathway (19, 58). Complement activation is necessary for a fully developed humoral immune response to PPS14 (30), but the role of complement in the response to polysaccharide conjugate vaccines has not previously been explored. We show here that complement activation is critical for a normal primary antibody response to both unmodified PPS14 and to PPS14-OVA conjugate in BALB/c mice. However, the secondary antibody response to both vaccines was enhanced following complement depletion at the time of primary immunization. Because the CVF-mediated effects on the secondary anti-PPS14 antibody response occurred after immunization with either unmodified PPS14 or the OVA conjugate, the effects of complement depletion were not simply a consequence of conjugation of a TD carrier to PPS14. Nonetheless, the secondary antibody responses to the two different vaccines still differed after CVF treatment. First, after PPS14 immunization, the magnitude of the CVF effect was much greater for IgM than IgG anti-PPS14 antibody concentrations. Second, postsecondary anti-PPS14 IgG concentrations were low with or without CVF treatment in mice immunized with PPS14. By contrast, CVF treatment resulted in increases in both IgM and IgG anti-PPS14 postsecondary antibody concentrations after immunization with PPS14-OVA, and the magnitude of the effect was much greater for anti-PPS14 IgG. We thus focused our additional studies on the effects of CVF on the immune response to PPS14-OVA conjugates.

Mice immunized with lower doses (0.2 and 0.5 μg) of PPS14-OVA had significantly enhanced anti-PPS14 antibody concentrations after secondary immunization, with the most significant increases in anti-PPS14 IgG occurring at the lowest dose of PPS14-OVA. The adjuvant effect of C3d conjugation on the humoral immune response to TD antigens is also most apparent at low doses of immunogen (11), suggesting that the effects of complement activation on different aspects of the adaptive immune response will be most easily detected when using lower immunizing doses of antigen.

IgG subclass switching and affinity maturation both occur within germinal centers (27, 49); thus, the differences we observed in IgG subclass distribution and avidity of IgG anti-PPS14 antibodies in mice treated with CVF likely reflect subtle changes in the germinal center reaction. IgG1 anti-PPS14 predominated after immunization with PPS14-OVA regardless of CVF treatment, but we did observe over a threefold increase in IgG3 anti-PPS14 after CVF treatment. Increases in IgG3 anti-polysaccharide antibodies have also been reported in mice immunized with PPS conjugate vaccines and CpG oligodeoxynucleotides (9) or meningococcal group C polysaccharide conjugate vaccines and interleukin-12 (4), suggesting that CVF treatment could affect the cytokine milieu within germinal centers. The magnitude of differences in IgG avidity in mice treated with CVF versus untreated mice was small, and this was reflected in the lack of difference in opsonophagocytic function of IgG1 antibodies from the two groups of mice. Overall, the functional attributes of anti-PPS14 IgG antibodies were largely unaffected by CVF treatment.

Experiments in which CVF was administered at different times relative to the time of immunization revealed that complement must be depleted during the first 2 to 4 days after primary immunization with PPS14-OVA to have an effect on the secondary anti-PPS14 antibody response. These findings are similar to those observed by Gray and coworkers, who found that treating mice with soluble CD40-γ1 within 4 days of immunization impaired the memory response to DNP-OVA but that treatment initiated later than 4 days after immunization was without effect (18). Indeed, the different effects of CVF treatment on the primary and memory responses to PPS14-OVA are remarkably similar to the effects of treatment of mice with stimulatory antibodies to CD27 or CD40 on the antibody response to TI-2 or TD antigens (42, 43). Ligation of CD27 or CD40 results in reduced expression of the transcription factor Blimp-1 (18, 43, 44), which plays a key role in inducing terminal differentiation of B cells into plasma cells (5). This suggests the possibility that complement-mediated effects could be occurring indirectly by affecting signaling via CD27 or CD40. Alternatively, complement could influence the plasma cell versus memory cell decision by independent mechanisms. Since it has been proposed that the strength of B-cell signaling may affect the memory cell versus plasma cell decision (5), it is relevant that coligation of CR2 and antigen receptors on B cells can lower both the concentration and affinity thresholds for B-cell activation (6, 34) and increase the level of B-cell activation for a given dose of antigen (6, 11, 53). Thus, complement activation at the time of immunization might help balance the B-cell fate decision in favor of plasma cells. Alternatively, complement activation might influence the differentiation of a specific memory B-cell precursor population (26) distinct from the cells responsible for generation of the primary antibody response.

The importance of the complement system in the memory response to TI-2 antigens has been subjected to minimal study to date. CVF given at the time of primary immunization decreased the secondary antibody response to high doses of particulate (39) or soluble (24) TD antigens, but the effects of CVF on the secondary response to low antigen doses or TI-2 antigens was not examined in either of those studies. Treatment with a monoclonal antibody that blocks ligand binding to both mouse CR1 and CR2 had no effect on the secondary antibody response to the TI-2 antigen Ficoll when given at the time of secondary immunization, but the effect of antibody given at the time of primary immunization was not examined (52). Mice treated with either a monoclonal antibody specific for CR1 only or one specific for both CR1 and CR2 had a diminished primary response to the TI-2 antigen dextran, but the secondary response was not measured (57). A decrease in the secondary response to both particulate and soluble T-cell-dependent antigens after treatment with the CR1/CR2-specific monoclonal antibody was shown to occur independent of any effect on T-cell priming (20), but whether this effect extends to T-cell help involved in the immune response to TI-2 antigens remains to be seen. Studies using a soluble form of CR2 to

block interactions between C3d(g) and CR2 have shown an inhibitory effect on the primary response to TD antigens in vivo (22), but analyses of its effects on the secondary response have only been performed in vitro (41). Finally, a number of studies have demonstrated a decreased secondary antibody response to TD, but not TI-2, antigens in mice lacking C3 (15, 16), C4 (15), or CR1 and CR2 (1, 10, 13, 29, 32). C3-deficient dogs had an impaired secondary antibody response to the TI-2 antigen DNP-Ficoll when immunized intravenously (38). The same study showed an enhanced secondary response to the TD antigen SRBC given intramuscularly but not intravenously, a result showing that the effects of complement on the humoral immune response may vary depending on the route of immunization. It should be noted that interactions of C3 fragments with their receptors would be permanently blocked in knockout mice or C3-deficient dogs, but only temporarily inhibited (e.g., at the time of primary immunization) in studies such as ours making use of CVF.

We previously showed that conjugation of C3d to PPS14 enhanced both the primary and secondary anti-PPS14 antibody responses relative to those induced by immunization with unmodified PPS14 and induced switching from IgM to IgG anti-PPS14 (51). The results of that study appear to conflict with those of the experiments reported here, where we found that complement depletion also resulted in an enhanced secondary anti-PPS14 antibody response. However, mice in our original report were immunized with PPS14-C3d at the time of both primary and secondary immunization, whereas mice demonstrating an increased secondary antibody response in this study were depleted of complement only at the time of primary immunization, leaving complement activation at the time of secondary immunization unaffected. These results are consistent with complement activation having dual effects on the humoral immune response to PPS14 or PPS14-OVA, enhancing both the immediate production of antibody via effects on interactions between the CD19/CD21/CD81 complex and B-cell receptor (14, 50), while at the same time having negative effects on the generation of memory B cells.

Our results also confirm reports that immunization with PPS conjugate vaccines can induce a strong antibody response in the absence of a spleen (3, 54). The means by which conjugation of TD carriers to PPS bypasses the requirement for the spleen in the anti-PPS antibody response is currently unknown, but likely involves enhancement of the anti-PPS14 immune response in extrasplenic locations. The effects of complement depletion were similar in normal and splenectomized mice immunized with PPS14-OVA but not unmodified PPS14, also suggesting the possibility that conjugation of a TD carrier to PPS14 influences the anatomic sites of memory B-cell development. However, the experiments reported here do not allow us to exclude the possibility that the immune response to PPS14-OVA ordinarily occurs primarily in the spleen and that conjugation of OVA simply enables other lymphoid tissues to mount an anti-PPS14 antibody response when the spleen is absent.

Our results may have practical implications with respect to the use of manipulations of complement to modulate the immune response to vaccines, particularly those making use of TI-2 antigens such as bacterial polysaccharides. Our results suggest that complement inhibition at the time of primary immunization could enhance the secondary (or subsequent) antibody response to vaccines consisting of either unmodified polysaccharide or polysaccharide conjugated to a protein carrier. Complement inhibition could be especially advantageous when used with conjugate vaccines, by allowing dosage reduction and extending vaccine supplies. Pneumococcal conjugate vaccines, which must contain conjugates of several different capsular polysaccharide serotypes, are more difficult to manufacture than monovalent vaccines. Shortages of the first licensed 7-valent pneumococcal conjugate vaccine have already been reported and in some cases have led to alterations in immunization schedules in both public and private immunization programs (17). More recently, elimination of the third and fourth doses of the pneumococcal conjugate vaccine in the initial immunization series have been recommended to maintain diminishing supplies of vaccine (7). Complement inhibition at the time of primary immunization might be an effective means of reducing overall vaccine dosage requirements by either permitting reduction in the amount of vaccine used in an individual injection or by decreasing the number of injections required to achieve protective antibody concentrations. Because the immune response differs among TI-2 antigens and between TI-2 and TD antigens (33), it is difficult to generalize our results to all immunogens. Thus, the clinical utility of complement inhibition as a means to enhance the immune response to vaccines will require extensive further study.

ACKNOWLEDGMENTS

We thank Emilee Bargoma for expert technical assistance and Alexander H. Lucas (Children's Hospital Oakland Research Institute) for the provision of monoclonal antibodies to PPS14.

This work was supported by National Institutes of Health grant AI-49250.

REFERENCES

1. **Ahearn, J. M., M. B. Fischer, D. Croix, S. Goerg, M. Ma, J. Xia, X. Zhou, R. G. Howard, T. L. Rothstein, and M. C. Carroll.** 1996. Disruption of the *CR2* locus results in a reduction in B-1a cells and in an impaired B cell response to T-dependent antigen. Immunity **4:**251–262.
2. **Artz, A. S., W. B. Ershler, and D. L. Longo.** 2003. Pneumococcal vaccination and revaccination of older adults. Clin. Microbiol. Rev. **16:**308–318.
3. **Breukels, M. A., A. Zandvoort, G. P. J. M. van den Dobbelsteen, A. van den Muijsenberg, M. E. Lodewijk, M. Beurret, P. A. Klok, W. Timens, and G. T. Rijkers.** 2001. Pneumococcal conjugate vaccines overcome the splenic dependency of antibody response to pneumococcal polysaccharides. Infect. Immun. **69:**7583–7587.
4. **Buchanan, R. M., D. E. Briles, B. P. Arulanandam, M. A. J. Westerink, R. H. Raeder, and D. W. Metzger.** 2001. IL-12-mediated increases in protection elicited by pneumococcal and meningococcal conjugate vaccines. Vaccine **19:**2020–2028.
5. **Calame, K. L.** 2001. Plasma cells: finding new light at the end of B cell development. Nat. Immunol. **2:**1103–1108.
6. **Carter, R. H., M. O. Spycher, Y. C. Ng, R. Hoffman, and D. T. Fearon.** 1988. Synergistic interaction between complement receptor 2 and membrane IgM on B lymphocytes. J. Immunol. **141:**457–463.
7. **Centers for Disease Control and Prevention.** 2004. Notice to readers: updated recommendations on the use of pneumococcal conjugate vaccine: suspension of recommendation for third and fourth dose. Morb. Mortal. Wkly. Rep. **53:**177–178.
8. **Chang, Q., Z. Zhong, A. Lees, M. Pekna, and L. Pirofski.** 2002. Structure-function relationships for human antibodies to pneumococcal capsular polysaccharide from transgenic mice with human immunoglobulin loci. Infect. Immun. **70:**4977–4986.
9. **Chu, R. S., T. McCool, N. S. Greenspan, J. R. Schreiber, and C. V. Harding.** 2000. CpG oligodeoxynucleotides act as adjuvants for pneumococcal polysaccharide-protein conjugate vaccines and enhance antipolysaccharide immunoglobulin G2a (IgG2a) and IgG3 antibodies. Infect. Immun. **68:**1450–1456.
10. **Croix, D. A., J. M. Ahearn, A. M. Rosengard, S. Han, G. Kelsoe, M. Ma, and M. C. Carroll.** 1996. Antibody response to a T-dependent antigen requires B cell expression of complement receptors. J. Exp. Med. **183:**1857–1864.
11. **Dempsey, P. W., M. E. D. Allison, S. Akkaraju, C. C. Goodnow, and D. T.**

Fearon. 1996. C3d of complement as a molecular adjuvant: bridging innate and acquired immunity. Science **271:**348–350.
12. **Dick, W. E., Jr., and M. Beurret.** 1989. Glycoconjugates of bacterial carbohydrate antigens. A survey and consideration of design and preparation factors. Contrib. Microbiol. Immunol. **10:**48–114.
13. **Fang, Y., C. Xu, Y.-X. Fu, V. M. Holers, and H. Molina.** 1998. Expression of complement receptors 1 and 2 on follicular dendritic cells is necessary for the generation of a strong antigen-specific IgG response. J. Immunol. **160:**5273–5279.
14. **Fearon, D. T., and R. H. Carter.** 1995. The CD19/CR2/TAPA-1 complex of B lymphocytes: linking natural to acquired immunity. Annu. Rev. Immunol. **13:**127–149.
15. **Fischer, M. B., M. Ma, S. Goerg, X. Zhou, J. Xia, O. Finco, S. Han, G. Kelsoe, R. G. Howard, T. L. Rothstein, E. Kremmer, F. S. Rosen, and M. C. Carroll.** 1996. Regulation of the B cell response to T-dependent antigens by classical pathway complement. J. Immunol. **157:**549–556.
16. **Fischer, M. B., M. Ma, N. C. Hsu, and M. C. Carroll.** 1998. Local synthesis of C3 within the splenic lymphoid compartment can reconstitute the impaired immune response in C3-deficient mice. J. Immunol. **160:**2619–2625.
17. **Freed, G. L., M. M. Davis, and S. J. Clark.** 2003. Variation in public and private supply of pneumococcal conjugate vaccine during a shortage. JAMA **289:**575–578.
18. **Gray, D., P. Dullforce, and S. Jainandunsing.** 1994. Memory B cell development but not germinal center formation is impaired by in vivo blockade of CD40-CD40 ligand interaction. J. Exp. Med. **180:**141–155.
19. **Griffioen, A. W., G. T. Rijkers, P. Janssens-Korpela, and B. J. M. Zegers.** 1991. Pneumococcal polysaccharides complexed with C3d bind to human B lymphocytes via complement receptor type 2. Infect. Immun. **29:**1839–1845.
20. **Gustavsson, S., T. Kinoshita, and B. Heyman.** 1995. Antibodies to murine complement receptor 1 and 2 can inhibit the antibody response in vivo without inhibiting T helper cell induction. J. Immunol. **154:**6524–6528.
21. **Hausdorff, W. P., J. Bryant, P. R. Paradiso, and G. R. Siber.** 2000. Which pneumococcal serogroups cause the most invasive disease: implications for conjugate vaccine formulation and use, part I. Clin. Infect. Dis. **30:**100–121.
22. **Hebell, T., J. M. Ahearn, and D. T. Fearon.** 1991. Suppression of the immune response by a soluble complement receptor of B lymphocytes. Science **254:**102–105.
23. **Immunization Practice Advisory Committee.** 1997. Prevention of pneumococcal disease: recommendation of the advisory committee on immunization practices (ACIP). Morb. Mortal. Wkly. Rep. **46**(RR-8)**:**1–24.
24. **Klaus, G. G. B., and J. H. Humphrey.** 1977. The generation of memory cells. I. The role of C3 in the generation of B memory cells. Immunology **33:**31–40.
25. **Klein, D. L., and R. W. Ellis.** 1997. Conjugate vaccines against *Streptococcus pneumoniae*, p. 503–525. *In* M. M. Levine, G. C. Woodrow, J. B. Kaper, and G. S. Cobon (ed.), New generation vaccines, 2nd ed. Marcel Dekker, Inc., New York, N.Y.
26. **Klinman, N. R.** 1997. The cellular origins of memory B cells. Semin. Immunol. **9:**241–247.
27. **Kosco-Vilbois, M. H., J.-Y. Bonnefoy, and Y. Chvatchko.** 1997. The physiology of murine germinal center reactions. Immunol. Rev. **156:**127–136.
28. **Lee, L. H., C.-J. Lee, and C. E. Frasch.** 2002. Development and evaluation of pneumococcal conjugate vaccines: clinical trials and control tests. Crit. Rev. Microbiol. **28:**27–41.
29. **Marchbank, K. J., C. C. Watson, D. F. Ritsema, and V. M. Holers.** 2000. Expression of human complement receptor 2 (CR2, CD21) in $Cr2^{-}/^{-}$ mice restores humoral immune function. J. Immunol. **165:**2354–2361.
30. **Markham, R. B., A. Nicholson-Weller, G. Schiffman, and D. L. Kasper.** 1982. The presence of sialic acid on two related bacterial polysaccharides determines the site of the primary immune response and the effect of complement depletion on the response in mice. J. Immunol. **128:**2731–2733.
31. **Martinelli, G. P., T. Matsuda, and A. G. Osler.** 1978. Studies of immunosuppression by cobra venom factor. I. On early IgG and IgM responses to sheep erythrocytes and DNP-protein conjugates. J. Immunol. **121:**2043–2047.
32. **Molina, H., V. M. Holers, B. Li, Y.-F. Fang, S. Mariathasan, J. Goellner, J. Strauss-Schoenberger, R. W. Karr, and D. D. Chaplin.** 1996. Markedly impaired humoral immune response in mice deficient in complement receptors 1 and 2. Proc. Natl. Acad. Sci. USA **93:**3357–3361.
33. **Mond, J. J., A. Lees, and C. M. Snapper.** 1995. T cell-independent antigens type 2. Annu. Rev. Immunol. **13:**655–692.
34. **Mongini, P. K. A., M. A. Vilensky, P. F. Highet, and J. K. Inman.** 1997. The affinity threshold for human B cell activation via the antigen receptor complex is reduced upon co-ligation of the antigen receptor with CD21 (CR2). J. Immunol. **159:**3782–3791.
35. **Monsigny, M., C. Petit, and A.-C. Roche.** 1988. Colorimetric determination of neutral sugars by a resorcinol sulfuric acid micromethod. Anal. Biochem. **175:**525–530.
36. **Mukherjee, J., A. Casadevall, and M. D. Scharff.** 1993. Molecular characterization of the humoral responses to *Cryptococcus neoformans* infection and glucuronoxylomannan-tetanus toxoid conjugate immunization. J. Exp. Med. **177:**1105–1116.
37. **Nieto, A., A. Gaya, M. Jansa, C. Moreno, and J. Vives.** 1984. Direct measurement of antibody affinity distribution by hapten-inhibition enzyme immunoassay. Mol. Immunol. **21:**537–543.
38. **O'Neil, K. M., H. D. Ochs, S. R. Heller, L. C. Cork, J. M. Morris, and J. A. Winkelstein.** 1988. Role of C3 in humoral immunity. Defective antibody production in C3-deficient dogs. J. Immunol. **140:**1939–1945.
39. **Pepys, M. B.** 1974. Role of complement in induction of antibody production in vivo. Effect of cobra venom factor and other C3-reactive agents on thymus-dependent and thymus-independent antibody responses. J. Exp. Med. **140:**126–145.
40. **Pepys, M. B.** 1975. Studies *in vivo* of cobra factor and murine C3. Immunology **28:**369–377.
41. **Qin, D., J. Wu, M. C. Carroll, G. F. Burton, A. K. Szakal, and J. G. Tew.** 1998. Evidence for an important interaction between a complement-derived CD21 ligand on follicular dendritic cells and CD21 on B cells in the initiation of IgG responses. J. Immunol. **161:**4549–4554.
42. **Raman, V. S., R. S. Akondy, S. Rath, V. Bal, and A. George.** 2003. Ligation of CD27 on B cells in vivo during primary immunization enhances commitment to memory B cell responses. J. Immunol. **171:**5876–5881.
43. **Raman, V. S., V. Bal, S. Rath, and A. George.** 2000. Ligation of CD27 on murine B cells responding to T-dependent and T-independent stimuli inhibits the generation of plasma cells. J. Immunol. **165:**6809–6815.
44. **Randall, T. D., A. W. Heath, L. Santos-Argumedo, M. C. Howard, I. L. Weissman, and F. E. Lund.** 1998. Arrest of B lymphocyte terminal differentiation by CD40 signaling: mechanism for lack of antibody-secreting cells in germinal centers. Immunity **8:**733–742.
45. **Rodriguez, M. E., G. P. J. M. van den Dobbelsteen, L. A. Oomen, O. de Weers, L. van Buren, M. Beurret, J. T. Poolman, and P. Hoogerhout.** 1998. Immunogenicity of *Streptococcus pneumoniae* type 6B and 14 polysaccharide-tetanus toxoid conjugates and the effect of uncoupled polysaccharide on the antigen-specific immune response. Vaccine **16:**1941–1949.
46. **Romero-Steiner, S., D. Libutti, L. B. Pasi, J. Dykes, P. Anderson, J. C. Whitin, H. L. Keyserling, and G. M. Carlone.** 1997. Standardization of an opsonophagocytic assay for the measurement of functional antibody activity against *Streptococcus pneumoniae* using differentiated HL-60 cells. Clin. Diagn. Lab. Immunol. **4:**415–422.
47. **Rubin, L. G.** 2000. Pneumococcal vaccine. Pediatr. Clin. North Am. **47:**269–285.
48. **Siber, G. R.** 1994. Pneumococcal disease: prospects for a new generation of vaccines. Science **265:**1385–1387.
49. **Stavnezer, J.** 1996. Immunoglobulin class switching. Curr. Opin. Immunol. **8:**199–205.
50. **Tedder, T. F., M. Inaoki, and S. Sato.** 1997. The CD19-CD21 complex regulates signal transduction thresholds governing humoral immunity and autoimmunity. Immunity **6:**107–118.
51. **Test, S. T., J. Mitsuyoshi, C. C. Connolly, and A. H. Lucas.** 2001. Increased immunogenicity and induction of class switching by conjugation of complement C3d to pneumococcal serotype 14 capsular polysaccharide. Infect. Immun. **69:**3031–3040.
52. **Thyphronitis, G., T. Kinoshita, K. Inoue, J. E. Schweinle, G. C. Tsokos, E. S. Metcalf, F. D. Finkelman, and J. E. Balow.** 1991. Modulation of mouse complement receptors 1 and 2 suppresses antibody responses in vivo. J. Immunol. **147:**224–230.
53. **Tsokos, G. C., J. D. Lambris, F. D. Finkelman, E. D. Anastassiou, and C. H. June.** 1990. Monovalent ligands of complement receptor 2 inhibit whereas polyvalent ligands enhance anti-Ig-induced human B cell intracytoplasmic free calcium concentration. J. Immunol. **144:**1640–1645.
54. **Van den Dobbelsteen, G. P. J. M., H. Kroes, and E. P. Van Rees.** 1995. Characteristics of immune responses to native and protein conjugated pneumococcal polysaccharide type 14. Scand. J. Immunol. **41:**273–280.
55. **Vogel, C.-W., R. Bredehorst, D. C. Fritzinger, T. Grunwald, P. Ziegelmüller, and M. A. Kock.** 1996. Structure and function of cobra venom factor, the complement-activating protein in cobra venom. Adv. Exp. Med. Biol. **391:**97–114.
56. **Whitney, C. G., M. M. Farley, J. Hadler, L. H. Harrison, N. M. Bennett, R. Lynfield, A. Reingold, P. R. Cieslak, T. Pilishvili, D. Jackson, R. R. Facklam, J. H. Jorgensen, and A. Schuchat.** 2003. Decline in invasive pneumococcal disease after the introduction of protein-polysaccharide conjugate vaccine. N. Engl. J. Med. **348:**1737–1746.
57. **Wiersma, E. J., T. Kinoshita, and B. Heyman.** 1991. Inhibition of immunological memory and T-independent humoral responses by monoclonal antibodies specific for murine complement receptors. Eur. J. Immunol. **21:**2501–2506.
58. **Winkelstein, J. A., J. A. Bocchini, Jr., and G. Schiffman.** 1976. The role of the capsular polysaccharide in the activation of the alternative pathway by the pneumococcus. J. Immunol. **116:**367–370.
59. **World Health Organization.** 1999. Pneumococcal vaccines. W.H.O. position paper. Wkly. Epidemiol. Rec. **74:**177–183.

Editor: J. N. Weiser